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Shear Stress, Temperature, and Inoculation Concentration Influence the Adhesion of Water-Stressed *Helicobacter pylori* to Stainless Steel 304 and Polypropylene

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Although molecular techniques have identified *Helicobacter pylori* in drinking water-associated biofilms, there is a lack of studies reporting what factors affect the attachment of the bacterium to plumbing materials. Therefore, the adhesion of *H. pylori* suspended in distilled water to stainless steel 304 (SS304) coupons placed on tissue culture plates subjected to different environmental conditions was monitored. The extent of adhesion was evaluated for different water exposure times, using epifluorescence microscopy to count total cell numbers. High shear stresses—estimated through computational fluid dynamics—negatively influenced the adhesion of *H. pylori* to the substrata ($P < 0.001$), a result that was confirmed in similar experiments with polypropylene ($P < 0.05$). However, the temperature and inoculation concentration appeared to have no effect on adhesion ($P > 0.05$). After 2 hours, *H. pylori* cells appeared to be isolated on the surface of SS304 and were able to form small aggregates with longer exposure times. However, the formation of a three-dimensional structure was only very rarely observed. This study suggests that the detection of the pathogen in well water described by other authors can be related to the increased ability of *H. pylori* to integrate into biofilms under conditions of low shear stress. It will also allow a more rational selection of locations to perform molecular or plate culture analysis for the detection of *H. pylori* in drinking water-associated biofilms.

Helicobacter pylori is the microorganism most frequently found in the human gastric mucosa in association with gastric epithelial cells (2). This gram-negative bacterium has a major role in promoting the risk of peptic ulcer disease and noncardia adenocarcinoma of the stomach, although new evidence is emerging that gastric *H. pylori* colonization has a protective role in relation to severe gastro-esophageal reflux disease and its sequelae, Barrett esophagus and adenocarcinoma of the esophagus (7). The prevalence of *H. pylori* infection in the world is assumed to be approximately 50%, with a higher prevalence in developing than in developed countries (11).

The route of transmission of *H. pylori* to humans remains controversial, with circumstantial evidence for infection via exposure to animals, contaminated water supplies, and oral reservoirs being reported previously (20). It has also been suggested that *H. pylori* may survive in water distribution networks by becoming associated with autochthonous microorganisms present in biofilms formed in such systems (5, 18, 29). Park et al. (22) reported the detection of *H. pylori* for the first time in drinking water biofilms taken from a public water distribution system. Other authors showed that *H. pylori* was capable of forming a monospecies biofilm (10, 26). However, these experiments were undertaken under high-nutrient conditions, which did not model the environment that *H. pylori* would encounter in a water distribution network. Because much of the research on *H. pylori* transmission in water is focused on detecting the bacterium in drinking water and associated biofilms, it is critical that a detailed description and explanation of the factors affecting the attachment of the bacterium to plumbing materials under low-nutrient conditions be undertaken.

So far, we have established that when a biofilm formed by a heterotrophic consortium of microorganisms is challenged with an *H. pylori* suspension, the pathogen tends to attach directly to the surface, preferably close to the basal layer of the biofilm (5). In this study, several different experimental conditions (temperature, shear stress, and inoculation concentration) were tested to determine their influence on the adhesion of water-exposed *H. pylori* to stainless steel 304 (SS304), one of the materials used in drinking water distribution systems.

MATERIALS AND METHODS

Culture maintenance. *H. pylori* NCTC 11637, obtained from Public Health Laboratory Services (Colindale, United Kingdom), was maintained on Columbia agar (Oxoid, Basingstoke, United Kingdom) supplemented with 5% (vol/vol) defibrinated horse blood (bioMérieux, Marcy l'Etoile, France). Plates were incubated at 37°C in a 2.5-liter Genbox jar (bioMérieux) under microaerophilic conditions created using a Genbox Microaer sachet (bioMérieux) and streaked onto fresh plates every 2 or 3 days.

Coupon preparation. Coupons measuring 2 by 2 cm were prepared at the Centro de Engenharia Biológica from stainless steel 304 (F. Ramada, Ovar, Portugal). For the purposes of washing and cleaning, a previously described procedure (8) was then applied, with some variations. Briefly, the material was immersed in a solution of commercial detergent (TopNeils; Tengelmann Portugal, Sintra, Portugal) and prewarmed distilled water for 30 min while it was gently mixed. To remove residual detergent, coupons were rinsed five times in ultrapure water, air dried, and wrapped in foil. SS304 coupons were autoclaved for 15 min at 121°C. Each coupon was finally placed in a well of a six-well tissue culture plate (Orange Scientific, Braine-l'Alleud, Belgium). For the experiment where polypropylene was used, all cleaning procedures were the same as those for SS304. However, polypropylene was only autoclaved at 80°C for 20 min.

Suspension preparation, inoculation, and culturable cell counts. For all conditions, cells from 2-day-old cultures were harvested from Columbia agar plates,

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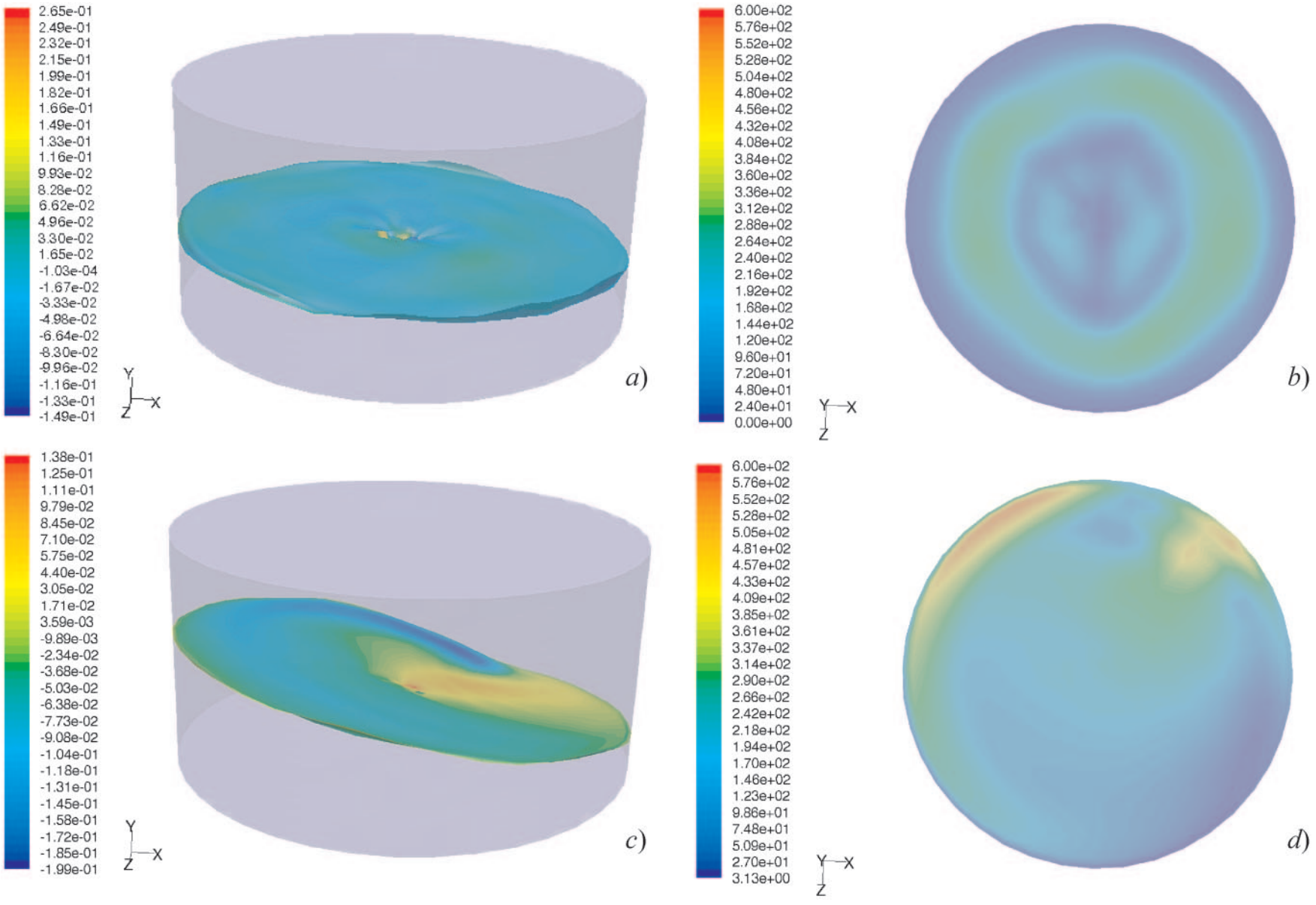

FIG. 1. Instantaneous results of numerical simulations using a three-dimensional unsteady volume-of-fluid laminar flow model in Fluent software. (a) Contours of liquid free surface at 60 rpm, colored according to tangential velocities (m/s). (b) Top view of contours of shear strain rate (1/s) 100 μm from the bottom of the well at 60 rpm. (c) Contours of liquid free surface at 120 rpm, colored according to tangential velocities (m/s). (d) Top view of contours of shear strain rate (1/s) 100 μm from the bottom of the well at 120 rpm.

suspended in 10 ml of autoclaved distilled water, and vortexed for 30 s. For the study of shear stress and temperature influence, this inoculum was transferred to a sterile flask containing 1,000 ml of autoclaved distilled water, with a final concentration of approximately 10^6 CFU/ml. The flask was maintained at room temperature (approximately 23 ± 1°C) and continuously stirred (120 rpm) using a magnetic bar. After 10 min, 5 ml of the suspended cells was dispensed into each of the wells of six-well tissue culture plates containing a coupon. The tissue culture plates were then placed either at various temperatures (4, 23, and 37°C) with no agitation or in orbital incubators (Certomat S; B. Braun Biotech International, Germany) set to 23°C at various agitation speeds (0, 60, and 120 rpm). The orbital diameter of agitation for this incubator was 14 mm.

For the study of the inoculum concentration influence, the bacteria were transferred to three different flasks to achieve final concentrations of 6.5×10^5, 2.5×10^6, and 6.9×10^6 CFU/ml. The *H. pylori* concentrations in the flasks were determined by plating 100 μl of the appropriate dilutions (1:10 dilutions in distilled water) onto three to five plates of HPSPA (27). Plates were incubated at 37°C for 6 days under the same microaerophilic conditions used for culture maintenance. The same procedure described for the other two experiments was then followed, with the tissue culture plates being stored at 23°C with no agitation.

For all experiments, one coupon for each condition was removed from the well at several times of exposure (2, 6, 12, 24, 48, 96, and 192 h), rinsed three times in autoclaved distilled water, and left to air dry for subsequent total cell count analysis.

Total cell counts of adhered bacteria. Fluorescent staining of bacteria was achieved by applying 80 μl of a 4′,6′-diamidino-2-phenylindole (DAPI; Sigma) solution (100 μg/ml) directly to the coupons for 5 min. The coupons were then rinsed in distilled water and left to air dry, and a drop of mounting oil was added to the surface. Finally, coupons were covered with coverslips and stored in the dark for up to 4 days. Cells were visualized under an epifluorescence microscope (Carl Zeiss, Germany) equipped with a filter sensitive to DAPI fluorescence. For each coupon, 50 fields were counted using an ocular grid, and the average of the results was expressed as total cells/cm^2. Depending on the number of squares of the ocular grid considered, the area of each field counted ranged from 0.004 to 0.025 mm^2.

Shear stress level prediction by numerical simulation. Because the standard approach for biofilm attachment studies involves the inclusion of a hydrodynamic parameter such as shear stress or flow speed, the shear stresses generated at each of the orbital incubator rotation speeds were estimated. Computational fluid dynamics software has been successfully applied in many research studies (e.g., see references 12, 24, and 30) and in nearly every manufacturing industry. It has become well established and validated for the study of simple air-liquid systems (e.g., see reference 3) and was therefore used to estimate the shear stress in this study.

Firstly, the geometry of the wells (each having a 34-mm diameter and a 17-mm height) was transposed into an equivalent grid size by computer. The geometry of the grid was fully three-dimensional, with a total of 23,000 structured, uniformly distributed, quadrilateral cells (finite volumes). Cell refinement was improved near the walls by a set of boundary layers. When wells were filled with 5 ml of water, the air-liquid interface was located about 5.5 mm from the bottom of the well, and thus the grid was separated into two fluid zones.

Liquid-airflow velocity fields were resolved by solving the full three-dimensional time-dependent (unsteady) Navier-Stokes equations with the commercial software package Fluent v6.1.22 (Fluent Inc., Lebanon, NH), using the volume of fluid multiphase model. Coupling of continuity and momentum equations was performed using the SIMPLE algorithm. The simulation model was started from a zero-velocity field. Simulations were run until the static pressures monitored at two points of the grid were repeated from one cycle to the next (i.e., a steady state was reached), which was normally achieved in 10 to 20 revolutions. The source momentum in the accelerating moving reference frame was specified by a user-defined function, which also updated the grid location at each time step.

From the local velocities obtained after the steady state was reached, the surface area-weighted average shear strain rate (γ) generated at the stationary adhesion surface in the bottom of the well was directly obtained. Shear stress (σ) was then calculated based on equation 1, where η is the dynamic viscosity of the fluid (considered in this case to be water at 23°C), as follows: $\sigma = \eta \times \gamma$.

Statistics. Total cell counts were transformed to the $\log_{10}$ scale. The steady state was considered to have occurred after 48 h (variation between the last three values for each condition never exceeded 5% between them), and averages were calculated based on the subsequent three values for each case. Averages were then compared using an appropriate statistical package (SPSS Inc., Chicago, IL) to perform a one-way analysis of variance followed by a Bonferroni post hoc test. Results were considered relevant for P values of <0.05.

TABLE 1. Relationship between rotation speed of orbital incubator, area-weighted average shear strain rate, and shear stress

Rotation speed (rpm)	Predicted area-weighted avg shear strain rate (1/s)	Shear stress (N/m^2)
0	0	0
60	146	0.138
120	334	0.317

RESULTS

By observing Fig. 1, it is clear that high shear strain levels are generated near the bottoms of the wells of tissue culture plates as a result of liquid sloshing: the higher the orbital frequency, the higher the centripetal force. As a consequence, higher shear strain rates (and shear stresses) exist in the surface where the cell tries to attach. Based on these results, the shear strain rate and shear stress for each condition were calculated (Table 1). Shear stress will be the term used for the rest of the article to reflect the different hydrodynamic conditions caused by the orbital incubator rotation speeds. The use of six-well tissue culture plates, coupled with appropriate mathematical analysis, is a convenient way to undertake many replicates of shear stress without having to use more demanding/tedious flow cells.

Total cell counts of attached *H. pylori* cells obtained for the different experiments and conditions are shown in Fig. 2. After only 2 hours, >5 $\log_{10}$ total cells/cm^2 were attached, regardless of the conditions tested. The temperature appeared to have no effect on the attachment of the pathogen (Fig. 2a), with most experimental points over time overlapping for the three temperatures. Decreasing shear forces, however, clearly promoted attachment, with an observable difference noticeable from the beginning of the experiment (Fig. 2b). Increasing inoculation densities appeared to have a proportional effect on attachment in the initial stages of the experiment, but not for the later stages (Fig. 2c).

To verify whether the adhesion of *H. pylori* to other materials was also affected by shear stress, a study of the adhesion of *H. pylori* to polypropylene at different shear stresses was also performed (Fig. 3). One-way analysis of variance followed by Bonferroni analysis confirmed that for different shear stresses at the steady state, the results were statistically significant between any of the combinations possible for SS304 ($P < 0.001$ in all cases) and for polypropylene ($P < 0.05$ in all cases). Using the same statistical approach, no such conclusions could be drawn about the effect of temperature or inoculation concentration on the adhesion of *H. pylori* to SS304 ($P > 0.05$).

After 6 hours, *H. pylori* cells appeared to be isolated on the surfaces of wells, and all three types of morphology (spiral, U-shaped, and coccoid) could be discriminated (Fig. 4). However, the cells were able to form small microcolonies (of <10 cells) after longer times of exposure. The appearance of a larger number of cocci or U-shaped bacteria with time was also observed. This behavior appeared not to be affected by the different conditions tested.

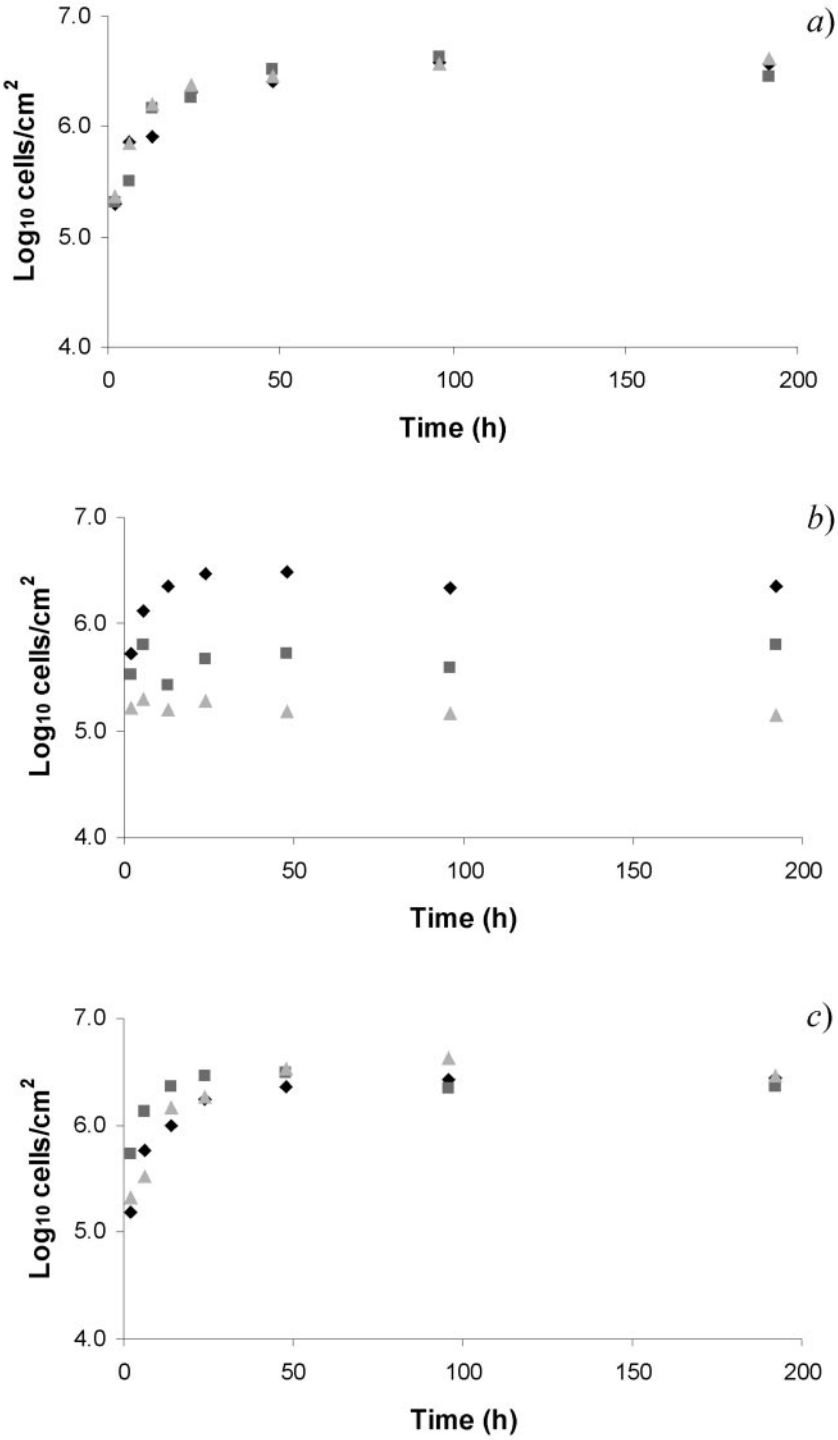

FIG. 2. Influence of different conditions on adhesion of *H. pylori* (NCTC 11637) to SS304. (a) Influence of temperature. ◆, 4°C; ▲, 23°C; ■, 37°C. (b) Influence of shear stress. ◆, 0 N/m^2; ■, 0.138 N/m^2; ▲, 0.317 N/m^2. (c) Influence of inoculation concentration, in $\log_{10}$ CFU/ml. ▲, 5.8; ◆, 6.4; ■, 6.8.

DISCUSSION

Some authors have reported the interference of shear stress levels of between 0.25 and 0.60 N/m^2 in laminar flow for the process of animal cell adhesion to surfaces (21). However, in contrast to the case with animal cells, some bacteria commonly present in water systems appear to have developed strategies to withstand high shear stresses while adhered. For instance, it is known that *Escherichia coli* can, under certain conditions, adhere more strongly to surfaces with increasing fluid velocities due to the action of the flagella (19) or of a lectin-like

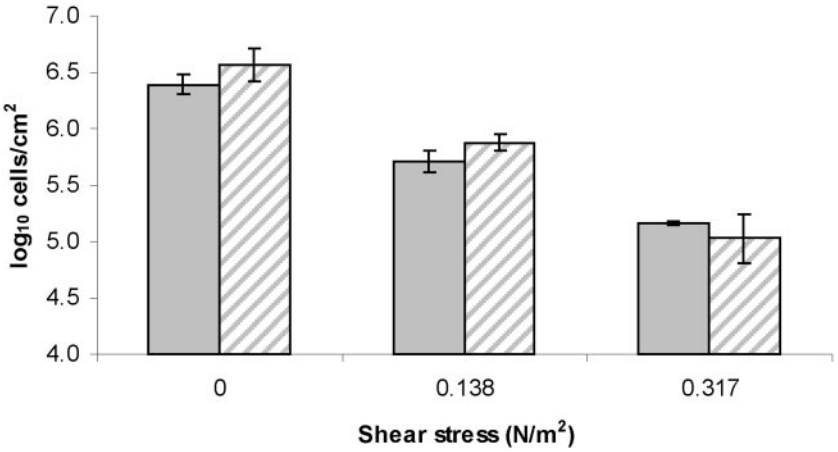

FIG. 3. Effect of shear stress on adhesion of *H. pylori* to SS304 (gray bars) and polypropylene (hatched bars). Each bar is the average of *H. pylori* adhered cell counts on three coupons taken after 48, 96, and 192 h ($n = 3$). Error bars represent standard deviations.

adhesin (28). These strategies might explain the results obtained by Percival et al. (23), where it was shown that higher viable and total cell counts of a heterotrophic consortium attached to slides of SS304 were obtained for higher flow rates (and, consequently, shear stresses) of water.

Although it is known that *H. pylori* expresses outer membrane-bound adhesins such as BabA, which is capable of interacting with the blood group antigen Lewis^b on gastric epithelial cells (14), this work shows that it lacks the necessary apparatus to strongly bind to abiotic surfaces when shear stress occurs. *H. pylori* should therefore be looked for in water storage reservoirs and well surfaces, where it is likely that low shear stresses and high residence times promote the attachment of the bacterium to surfaces and, consequently, an association with biofilms. In fact, one study found a positive relationship between *H. pylori* infection and well water (15), and others reported the detection of the pathogen in these types of systems by using molecular methods (6, 13, 17). Although one of the reasons pointed out was the lack of chemical treatment of the water, this work indicates that it can also be related to the increased ability of the bacteria to integrate into biofilms under these conditions.

In contrast to our expectations, there was no observable effect of temperature on the adhesion of *H. pylori*. It has been described that the bacterium remains culturable for much longer in water at low temperatures (22 days at 4°C) than at high temperatures (6 to 10 h at 23°C) (1, 4, 25). The results of culturability tests performed on planktonic cells in one of our experiments at 23°C agreed with this observation, with a loss of culturability being observed after 6 h (N. F. Azevedo, M. J. Vieira, and C. W. Keevil, unpublished data). It has also been proposed that the attachment and persistence of *H. pylori* in surfaces/biofilms are phenomena requiring the organism to be in a viable state (18). If this is the case, and because the length of our experiment was 7 days, this work also suggests that the bacterium is in a viable but nonculturable state at higher temperatures.

The data obtained about the influence of inoculation concen-

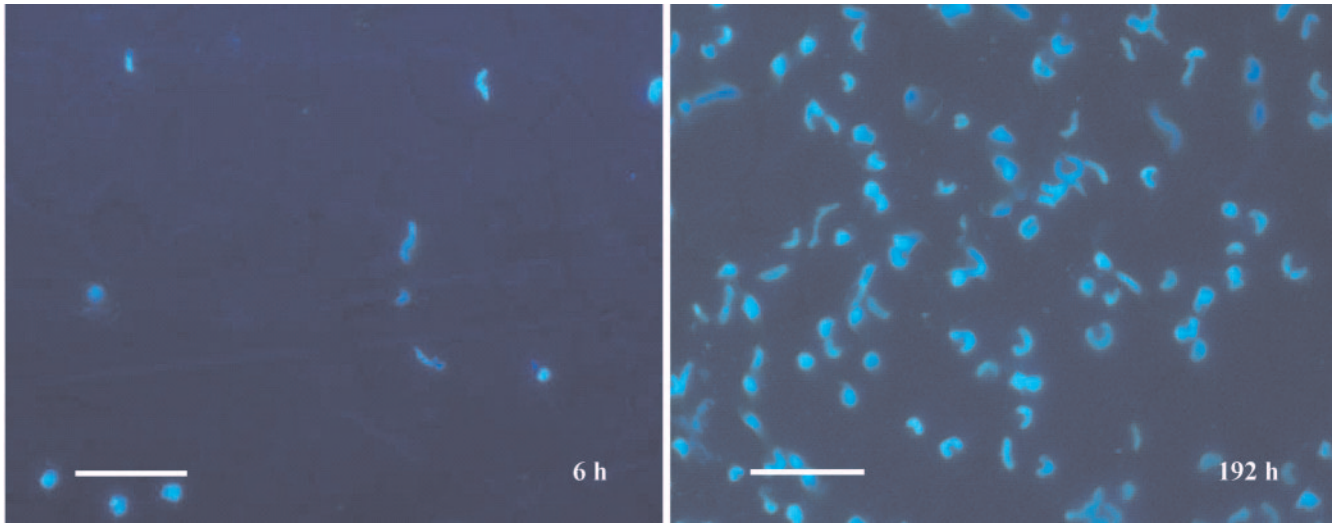

FIG. 4. Fluorescence microscopy images showing adhesion of *H. pylori* to SS304 after 6 and 192 h at 23°C with no shear stress. Bar, 10 μm.

tration are in agreement with work presented by Cerca et al. (9), where it is stated that for each condition, there is a maximum number of bacterial cells that can adhere to a surface, regardless of the initial cellular concentration used. This implies that concentrations of 6.5×10^5 CFU/ml and perhaps even lower will have the same effect in terms of bacterial attachment as long as sufficient contact time between the two surfaces is established.

When comparing the kinetics of *H. pylori* adhesion with the results obtained by Cole et al. (10), we observed that *H. pylori* adhesion also begins with individual bacteria adhering to the abiotic surface, with subsequent expansion into colonies. However, the formation of three-dimensional structures at the air-liquid interface described in that work was only very rarely observed. This might happen because (i) *H. pylori* is unable to divide under low nutrient conditions and hence not capable of consistently forming such structures, (ii) longer times are required for *H. pylori* to form these structures under these conditions, or (iii) *H. pylori* can only form these structures at the air-liquid interfaces. The formation of three-dimensional structures usually creates microenvironments where the bacteria have better chances of survival (16). The apparent inability of *H. pylori* to do so implies that either adhesion conditions not tested in this study or coaggregation with other microorganisms might be necessary. In fact, free-living amoebae, which are typical predators found in biofilm ecosystems, were already found to provide conditions favoring the survival of *H. pylori* (29), and we have also previously shown that *H. pylori* subsists close to the basal layer of heterotrophic biofilms, possibly benefiting from the low redox zones created by these structures to enhance survival (5).

This study represents, to the best of our knowledge, the first report on the effect of different conditions on the adhesion of *H. pylori* to a pipe material. The study indicates that the frequent detection of the pathogen in well water by other authors can be related to the increased ability of the bacteria to be incorporated into biofilms under conditions of low shear stress. It will also lead researchers to perform a more rational selection of locations to perform molecular or plate culture analysis for the detection of sessile *H. pylori* cells.

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